EX. C

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Borbala Gesser and Christian Grønhøj Larsen
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For	:	Immunomadulators
Examiner	:	D. Basham
Art unit	:	1812

Declaration of Borbala Gesser

1. I, Borbala Gesser, Pilegårdsvej 233, DK-8361 Hasselager, Kolt, Denmark, who am an assistent professor at Marselisborg Hospital, DK-8000 Aarhus C, Denmark, do state and declare as follows:

2. I am one of the named inventors of the above-captioned patent application. I believe that I am a person skilled in the art to which the above-captioned application pertains.

3. a) I have tested the twelve peptides outlined below for their biological activity with respect to the induction of IRAP in a monocyte cell culture (property c) mentioned in the above-captioned patent application e.g. at page 4) in order to study variants of the peptide IT9302.

3. b) Furthermore, a peptide wherein X_4 is Ile was tested for the production of IL-8 by normal human peripheral blood mononuclear cells (PBMC) corresponding to property b) in the patent application.

4. Materials and methods

The modified peptides were synthesized by Schafer-N, Fruebjergvej 3, DK-2100 Copenhagen, Denmark. Stock solutions were made from each peptide by dissolving 1 mg peptide in 1 ml phosphate buffer saline pH 8.0 with 4% BSA (bovine serum albumin). rIl-10 was purchased from R&D systems Europe Ltd, 4-10 The Quadrani, Barton Lane, Abingdon, Oxon Ox143YS UK. The preparation of rIl-10 from the supplier contained BSA. rIL-10 and IT9302 were used as controls. The stock solutions of the peptides were diluted so as they were equimolar to rIL-10.

IRAP induction by the modified peptides, IT9302 and rIL-10 in purified monocytes was performed essentially as described in example 3 at page 48 and at page 44-45 in the above-captioned patent application. Blood from 2 normal healthy donors was used, each donor gave 200 ml blood, and the yield of monocytes was 33 and 39 million cells, respectively, per donor. During the purification the cells are kept at 4°C (on ice) to

avoid stimulation, which can result in endogenous IRAP. Monocytes was separated from the heparinized blood within 1 to 2 hours. For stimulation it is possible to use 1 million or 2 million sells per ml cell culture media (RPMI 1640, GIBCO, cat. no. 6187-010)). Cells were stimulated with 1, 10 and 100 ng peptides per ml (equimolar to IL-10) or rIL-10 per ml media. Cells were stimulated overnight and the IRAP induction was measured in the supernatants by ELISA (R&D System Europe Ltd).

Furthemore, for the peptide having X_4 Met substituted by Ile the test for the production of IL-8 by normal human peripheral blood mononuclear cells (PBMC) corresponding to property b) was performed essentially as described at pages 43-44 in the above-captioned patent application. The amount of peptides tested were adjusted in equimolar amounts to hIl-10 as follows 2µg/ml, 200ng/ml, 20ng/ml and 2ng/ml.

5. Results

The original peptide IT9302 has the sequence:

Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn

Re a) The tested twelve peptides have the sequences:

	X_1 X_2 X_3 X_4 X_5 X_6	
Mod-1:	Ala-Phe-Met-Thr-Leu-Lys-Leu-Arg-Asn (SEQ ID NO:3)	+
Mod-2:	Ala-Tyr-Met-Thr-Met-Lys-Val-Arg-Glu (SEQ ID NO:4)	-
Mod-3:	Gly-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn (SEQ ID NO:5)	+
Mod-4:	Ala-Phe-Met-Thr-Met-Lys-Ile-Arg-Asn (SEQ ID NO:6)	-
Mod-5:	Ala-Tyr-Ile-Thr-Met-Lys-Ile-Arg-Gln (SEQ ID NO:7)	+
Mod-6:	Ala-Tyr-Leu-Thr-Met-Lys-Ile-Arg-Asn (SEQ ID NO:8)	-
Mod-7:	Ala-Tyr-Val-Thr-Met-Lys-Ile-Arg-Asn (SEQ ID NO:9)	-
Mod-8:	Ala-Tyr-Met-Thr-Leu-Lys-Ile-Arg-Asn (SEQ ID NO:11)	-
Mod-9:	Ala-Tyr-Met-Thr-Val-Lys-Ile-Arg-Asn (SEQ ID NO:12)	-
Mod-10:	Ala-Tyr-Met-Thr-Met-Lys-Val-Arg-Asn (SEQ ID NO:15)	+
Mod-11:	Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Gln (SEQ ID NO:16)	+
Mod-12:	Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Glu (SEQ ID NO: 17)	-

The modifications marked with "+" posses the measured biological activity, whereas the modifications marked with "-" had no activity.

The above results (+/-, respectively) is the conclusion of the following test results:

Experiment 1

1,8 million monocytes per ml

Added peptide equimolar concentration to IL-10 (100 ng/ml)	Induced IRAP (ng/ml)
Control cells without addition of peptide	20.5 +/-1.5
IT9302	30.0 +/- 6.0
IL-10	20.5 +/- 1,5
Mod-1	28.0 +/- 3.0
Mod-2	20.0 +/- 0
Mod-3	27.5 +/- 1.5
Mod-4	19.5 +/- 1.5
Mod-5	28.0 +/- 7.0
Mod-6	24.0 +/- 3.0
Mod-7	21.5 +/- 0.5
Mod-8	19.5 +/- 1.5
Mod-9	21.0 +/- 1.0
Mod-10	24.0 +/- 3.0
Mod-11	16.0 +/- 0.5
Mod-12	20.0 +/- 1.0

Experiment 2

1 million monocytes
per ml

Control cells without addition of peptide	23.0 +/- 1.0 25.0 +/- 4.0
IT9302 100 ng/ml	30.0 +/- 3.0
IL-10 100 ng/ml	35.0 +/- 5.0
Mod-1	
100 ng/ml	25.0 +/- 2.0
10 ng/ml	26.0 +/- 4.0
1 ng/ml	30.0 +/- 1.0
Mod-3	
100 ng/ml	27.0 +/- 3.0
10 ng/ml	27.0 +/- 1.0
1 ng/ml	27.5 +/- 0,5
Mod-5	
100 ng/ml	30.0 +/- 3.0
10 ng/ml	41.0 +/- 0.5
1 ng/ml	38.0 +/- 2.0
Mod-6 100 ng/ml	22.5 -/- 3.5
Mod-8 100 ng/ml	21.0 -/- 0.0
Mod-10	32.0 +/- 1.0
Mod-11	
100 ng/ml	32.0 +/- 1.0
10 ng/ml	24.0 +/- 0.0
1 ng/ml	16.0 +/- 3.0

Re b) It was found that 50% inhibition of production of IL-8 by normal human peripheral blood mononuclear cells (PBMC) was achieved with 20 or 200 ng for modified peptide Ala-Tyr-Met-Thr-Ile--Lys-Ile-Arg-Asn. This result was similar to the effect on IL-8 production of IT9302.

6. Discussion

These results show that it indeed is possible to mutate the original peptide by as many as three different mutations (Mod-1) and still retain activity c). It is noted that even though both Mod-1 and Mod-8 have Leu in position X_4, Mod-1 had activity and Mod-8 had not. One explanation for this phenomenon could be of configuration reasons and that it ap-

parently seems like it is possible to compensate for some perturbations, both negatively by themselves (Mod-8 (Y2F) and Mod-9 (M5L) with Mod-1 (I7L)). Similarly is seen for Val in position X_5 where Mod-10 had the measured biological activity and Mod-2 had not. These results show that whether a particular amino acid in a given position in fact confers the desired property to the peptide actually depends on the identity of the rest of the amino acids in the peptide. For that reason it would not be proper, in my view, to conclude that because the substitution of X_3 for Leu and Val, respectively, did not show any activity in the present study, then Leu and Val in the X_3 position will never work. Furthermore, it is noted that only one activity c) was tested for the twelve peptides and hence it is possible that the modifications which did not retain activity c) could posses one or more of the biological actitity outlined in the above-captioned application.

It is my opinion that the application enables the person skilled in the art to test the proposed peptides for the induction of IRAP in monocyte cells culture. Any research group who is trained in purifying monocytes from heparinized fresh blood by plastic adherence technique, can perform this. The above test was done twice for each peptide with monocytes from two different person for statistical reasons. It took about 82 hours for one person to test the above 12 peptides (duration was 2 weeks - i.e. about 7 hours per peptide). It took 6 weeks for Schafner to deliver the 12 peptides. A portion of 5 mg of each peptide were synthesized for a cost of 3300 Dkr plus 25 % taxes for each peptide. About 1 mg of each peptide is needed for the above test. The cost of two Quatikine IL-1 receptor antagonist DRAOO Kit is 7840.00 Dkr. plus 25 % taxes.

Finally, in view of the foregoing, it would not require undue experimentation to systematically determine the effect of all possible 64 combinations of X_4, X_5, and X_6 on biological activity.

7. I further declare that all statements made herein of my own knowledge are true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 30.9.98 Signature: Borbala Gesser

Borbala Gesser